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Effect of anchor and core sequence in microsatellite primers on flax fingerprinting patterns

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SUMMARY

The aim of this study was to select the best arrangements of MP–PCR (microsatellite-primed PCR) for routine large-scale fingerprinting of flax cultivars. We found optimum PCR conditions for the application of five previously published primers (PCT1–PCT5) to flax cultivar fingerprinting. We modified to optimum MP–PCR which was targeted to flax tetrameric [GATA] microsatellite loci specified by primer PCT6. We found that after a reamplification PCR step was involved we were able to generate highly discriminating fingerprinting patterns, which distinguished all eight flax cultivars individually. In particular primers 3PCT1 and 3PCT2 were promising for future large-scale fingerprinting due to the production of most polymorphic bands. Increasing annealing temperature within a temperature profile helped to generate new polymorphisms within flax microsatellite patterns especially with primer 3PCT2. Using this primer we succeeded in generating new polymorphic bands after increasing annealing temperature from 55 °C to 60 °C, and to 65 °C. A cluster analysis of flax cultivars was performed based on microsatellite data. The core group of eight flax cultivars was clustered into two homogeneous subclusters. A lower level of cultivar clustering within subclusters was not detected.

INTRODUCTION

The identification of flax varieties is currently based mainly on morphological characters which often do not enable clear decisions to be made. Therefore molecular fingerprinting techniques are becoming more important as a tool for cultivar or varietal identification in plant breeding and germplasm management (Smith 1998). Increasing acceptance of molecular markers is evident in a recent court case where DNA markers were accepted as evidence with regard to patented varieties (Congiu *et al*. 2000).

Microsatellites or simple sequence repeats (SSR) have proved to be a highly informative DNA marker system in plants because of a high degree of polymorphism, codominant mode of inheritance and simple data interpretation (Thomas *et al*. 1994). Inter-SSR fingerprinting has been successful in a wide range of agronomically important crops such as maize or oil seed rape (Kantety *et al*. 1995; Charters *et al*. 1996). An effective alternative to the traditional but very laborious approach of using specific PCR (polymerase chain reaction) primers based on microsatellite-flanking sequences is microsatellite fingerprinting which involves the use of simple sequence repeat (SSR) primers in PCR amplification (Morgante & Olivieri 1993; Gupta *et al*. 1994; Weising *et al*. 1995). Microsatellite-primed PCR (MP–PCR) (Vogel & Scolnik 1998) with anchored SSR primers, also called Inter-SSR (ISSR) PCR (Zietkiewicz *et al*. 1994), is based on closely spaced SSR loci oppositely oriented on the DNA template. These loci serve thereafter as primer binding sites to initiate amplification of the intervening DNA sequences of different sizes. MP–PCR is more reproducible and produces better polymorphism patterns than the older but frequently used random amplified polymorphic DNA (RAPD) approach (Siffelova *et al*. 1997, 1998). SSR loci often produce high levels of DNA polymorphism per locus within a genome (Smith *et al*. 1997).

As far as flax is concerned, isozyme systems were generated only recently (Gorman *et al*. 1993) and, in comparison to other crop genomes, the development of DNA marker systems is in an initial phase. RAPD polymorphism was studied in seven newly generated flax rust resistance lines to monitor introgression and selected RAPD markers were cloned to produce

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restriction fragment length polymorphisms (RFLPs) (Cullis & Govindaraju 1992). Flax RAPDs were further optimized by Aldrich & Cullis (1993). DNA markers for *Fusarium* wilt resistance genes for the low linolenic LINOLA were searched for by applying bulked segregant analysis but only weak genetic linkage was found (Spielmeyer *et al*. 1995). DNA markers for *Fusarium* associated with low linolenic mutations were also detected.

An initial version of the flax genetic map was constructed using segregation data from 16 RAPD, 22 RFLP, 4 isozyme and 4 morphological polymorphisms giving an estimated 185 kb per cM (Gorman & Parojcic 1992). Nineteen RFLP and 69 RAPD markers have been mapped onto 15 linkage groups based on an F_2 mapping population from the crosses of CI 1303 × Stormont Citrus (Cullis *et al*. 1995).

More recently, an amplified fragment length polymorphism (AFLP) genetic linkage map of flax was constructed to identify quantitative loci with major effect on resistance to *Fusarium* genes (Spielmeyer *et al*. 1998). Here a set of 213 AFLP marker loci covered approximately 1400 cM of the flax genome ($n = 15$) comprising 18 linkage groups with an average spacing of 10 cM, which represents a moderately saturated map. This map also included some RFLP markers linked to *Melampsora* resistance. Another linkage analysis based on RAPD and AFLP markers resulted in 19 linkage groups with an average of 11·6 cM distance between markers (Klocke *et al*. 2000).

In the study reported here, we have developed MP–PCR (ISSR) technology for routine fingerprinting of the flax genome. The method was made suitable for subsequent large-scale fingerprinting of flax cultivars. We focused particularly on selection and testing of already published microsatellite primers, design of MP–PCR primers *de novo*, optimization of PCR thermoprofiles, selection of the best type of thermostabile DNA polymerase, and on testing the effects of various agarose gel formulae.

Table 1. *The list of screened flax cultivars*

Linum usitatissimum L. cultivars	Country of origin	Industrial type
LAURA	The Netherlands	Fibre
JORDAN	Czech Republic	Fibre
VENIKA	Czech Republic	Fibre
WIKO	Poland	Fibre
BILTSTAR	The Netherlands	Oil
ARIANE	France	Fibre
BONET	Czech Republic	Fibre
JITKA	Czech Republic	Fibre

Table 2. *Sequences of microsatellite-specific PCR primers used in MP–PCR*

Primer	Sequence
PCT1	5′-KKY HYH Y$(GA)_{15}$
PCT2	5′-KKV RVR V$(CT)_{15}$
PCT3	5′-KKY NSSH$(ATG)_6$
PCT4	5′-KKV RVR V$(CT)_6$
PCT5	5′-KKV RVR V$(TG)_6$
PCT6	5′-KKB NVS S$(GATA)_6$
3PCT1	5′-YHY$(GA)_{15}$
3PCT2	5′-VRV$(CT)_{15}$
3PCT3	5′-SSH$(ATG)_6$
3PCT4	5′-VRV$(CT)_6$
3PCT5	5′-VRV$(TG)_6$
3PCT6	5′-VSS$(GATA)_6$
3PCT1S	5′-YHY$(GA)_6$
3PCT4L	5′-VRV$(CT)_{15}$
3PCT5L	5′-VRV$(TG)_{15}$
3PCTanS	5′-SSS$(GA)_6$
3PCTanW	5′-WWW$(GA)_6$
PCT1an3W	5′-$(GA)_{15}$WWT
PCT1an3S	5′-$(GA)_{15}$SSG
PCT2an3W	5′-$(CT)_{15}$WWT
PCT2an3S	5′-$(CT)_{15}$SSG
PCT1noan	5′-$(GA)_{15}$
PCT2noan	5′-$(CT)_{15}$
PCTAT	5′-N7$(AT)_{10}$
PCTAA	5′-N7$(A)_{20}$

MATERIAL AND METHODS

The flax cultivars listed in Table 1 were obtained from AGRITEC Research, Breeding, Services Ltd. Šumperk, Czech Republic and from Sempra Prague, Breeding Station Slapy u Tabora.

DNA samples were extracted from 1-week-old seedlings germinated on filter paper in a dark box following the modified DNA extraction protocol of Cullings (1992). DNA of the individual genotypes was also extracted from the roots of grown plants. At least two independent DNA samples were analysed for each cultivar genotype.

Microsatellite-primed PCR (MP–PCR, Vogel & Skolnik 1998) was used to generate fingerprinting patterns to distinguish among flax cultivars. Oligonucleotide primers were synthesized by Generi (Hradec Kralove, CR, for sequences see Table 2) using sequences suggested in Fisher *et al*. (1996) and Brachet *et al*. (1999), and using sequences designed *de novo*. Specifically, we varied the following characteristics of a MS-primer:

(a) anchor length
(b) 5′ or 3′ anchor position
(c) anchor sequence
(d) length of core sequence
(e) core sequence.

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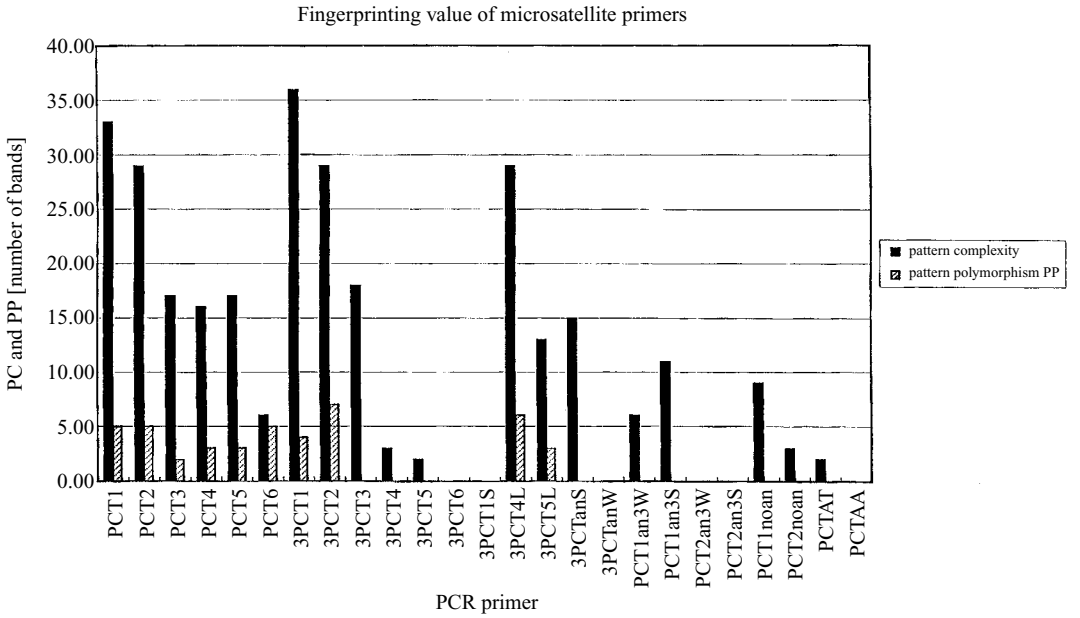

Fig. 1. Fingerprinting value of flax microsatellite primers. *PC* parameter (■) corresponds to the total number of microsatellite bands per electrophoretic pattern, *PP* parameter (▨) corresponds to the number of polymorphic (cultivar – discriminative) microsatellite bands per electrophoretic pattern.

PCR reaction mixture (25 µL volume) contained 25 µL 10 × PCR buffer, 4 mM $MgCl_2$, 2·5 mM dNTP mix, 0·2 µM primer, 20 ng template DNA and 1U TaKaRa polymerase (TaKaRa Shuzo, Japan). PCR thermocycling was carried out on a Perkin–Elmer 480 thermal cycler using the following profile: 1 cycle of 94 °C for 3 min followed by 35 cycles of 94 °C for 60 s, an annealing temperature optionally of 55 °C, 60 °C or 65 °C for 30 s, followed by 72 °C for 30 s finished by 1 cycle of 72 °C for 3 min. Amplification products were separated on 1·5% agarose/TAE gels with ethidium bromide visualization. DNA ladder 100 bp (New England BioLabs, USA) was used as a DNA size marker.

The parameter of polymorphism information content (*PIC*) was calculated according to Botstein *et al.* (1980): $PIC_j = n(1-\Sigma P_{ij}^2)/(n-1)$ where n is the sample size, and P_{ij} the frequency of the i-th pattern revealed by the microsatellite primer j summed across all patterns revealed by the primer j.

For clear interpretation of patterns we defined two further parameters:

(a) Pattern complexity – *PC*
PC_n … sum of all scorable bands per electrophoretic pattern n, and related *PC*-derived parameters:
PC_a … average *PC* per set of patterns
PC_t … total *PC*, total sum of all scorable bands per set of patterns.

(b) Pattern polymorphism (i.e. total number of polymorphic bands) – *PP*
PP_n … sum of scorable polymorphic bands per electrophoretic pattern n, i.e. the sum of bands which differentiate among screened flax cultivars, and related *PP*-derived parameters:
PP_a … average *PP* per set of patterns
PP_t … total *PP*, total sum of all scorable polymorphic bands per set of patterns.

The cluster analysis of electrophoretic data was calculated by GelCompar II, version 2·0 software package (Applied Maths., Kortrijk, Belgium), from which a variant of the Pearson correlation coefficient and the UPGMA clustering procedure were selected.

RESULTS AND DISCUSSION

First, we compared various types of DNA thermostable polymerase. We compared TaKaRa Taq DNA polymerase (Takara Shuzo Co., Japan), AGS Gold DNA polymerase (Hybaid, UK), and DYNAzyme II DNA polymerase (Finnzymes OY, Finland). In general, no exploitable differences were found between TaKaRa and Gold, while DYNAzyme produced weaker patterns with impoverished longer amplicons indicating lower processivity. We used TaKaRa DNA polymerase in further experiments.

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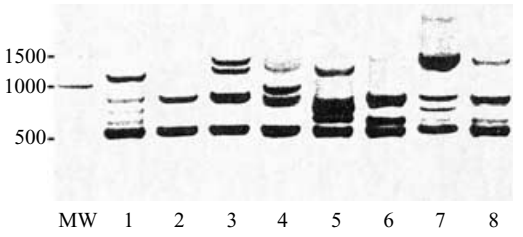

Fig. 2. Effect of reamplification: enhancement of PCR signal using MS primer PCT6 (5′-KKB NVS S[GATA]$_6$) on the microsatellite pattern for the eight flax cultivars. The single-run PCR gave completely empty patterns while after reamplification using aliquot from the first run highly polymorphic patterns with moderate complexity were obtained which were able to distinguish all eight flax cultivars completely.

The various types of agaroses we tested comprised agarose (FMC BioProducts, USA), agarose, molecular-biology-grade (Promega Co., USA), and agarose, wide range (Sigma, USA). No substantial impact of agarose type on quality of electrophoretic pattern was found even though MetaPhor agarose was reported by the manufacturer to approach the high resolution of polyacrylamide gels. We used the Sigma agarose in further experiments.

We started testing MS primers using a basic set published by Fisher *et al.* (1996), and Brachet *et al.* (1999), i.e. the set of MS primers listed as PCT1 to PCT6 (Table 2) which were originally applied to the common ash genome (*Fraxinus excelsior*). The essential variation in this 'Brachet' group of primers is in core sequence with a constant anchor length (characteristic *e*) in the *Material and Methods*). A summary of the *PC* and *PP* parameters for the fingerprinting patterns generated is given in Fig. 1. Clearly, this group of primers was also effective in the flax genome for the production of bands (*PC*) and in demonstrating band polymorphism (*PP*). The original 'Brachet' group of primers together with a modified (by us) PCR with PCT6 primer (see below) gave total *PC*: $PC_t = 118$ and total *PP*: $PP_t = 23$ with the following simple statistics: average pattern complexity *PC*: $PC_a = 19{\cdot}67$ (var. coeff. = 49·8 %) and average pattern polymorphism $PP_a = 3{\cdot}8$ (var. coeff. = 34·7 %).

Longer sequence motifs (like $[GATA]_n$ in PCT6 primer) should, by simple probability statistics, have a lower frequency within a genome and therefore could be less productive for routine MP–PCR screening. Indeed the series of primers specific to microsatellites (MS) with longer core motifs usually gave empty fingerprinting patterns in our experiments. We also obtained a completely empty pattern when amplified tetrameric [GATA] microsatellite loci specified by primer PCT6 was used in ordinary single-run PCR. However, when a reamplification step was inserted we generated the most potent fingerprinting pattern we have ever found in flax (Fig. 2). From this result we hypothesize that microsatellite loci of longer core sequence represent too high a challenge for ordinary polymerase processivity and result therefore in empty patterns after the single-run PCR. No signal on the agarose gel may be the consequence of too low a concentration of amplified products being below the detection threshold defined by ethidium bromide sensitivity. It appears that this may be satisfactorily compensated for by insertion of a PCR reamplification step. Fingerprinting patterns obtained in this way may then provide much more information than the commonly used short core MS primers. This finding justifies focusing future fingerprinting experiments on tetra-, penta-, and hexameric core sequences with exploitation of reamplification strategy.

Further, we compared the group of MS primers with [GA] core sequence to a complementary group of [CT] core sequence. The [GA] group gave $PC_t = 110$ and $PP_t = 9$ with $PC_a = 13{\cdot}7$ (var. coeff. = 100·5 %) and $PP_a = 1{\cdot}13$ (var. coeff. = 186·7 %), while the [CT] group gave $PC_t = 109$ and $PP_t = 21$ with $PC_a = 13{\cdot}6$ (var. coeff. = 100·5 %), and $PP_a = 2{\cdot}6$ (var. coeff. = 115·17 %). Interestingly the [CT] primer gave more than twice as many polymorphic bands as [GA] which suggests amplification of different inter-microsatellite loci in the flax genome. A high value of variation coefficient reflects substantial differences between fingerprinting patterns of individual cultivars.

We designed a group of primers with truncated anchors to study the effect of a short anchor (primers 3PCT1 to 3PCT6, Table 2). This group produced together $PC_t = 88$ and $PP_t = 11$ with $PC_a = 4{\cdot}7$ (var. coeff. = 104·9 %), and $PP_a = 1{\cdot}8$ (var. coeff. = 163·3 %). For fingerprinting large cultivar sets primers 3PCT1 and 3PCT2 should be particularly promising in the production of polymorphic bands (high *PP* value, Fig. 1).

We also compared the effect of decreasing anchor length. The tendencies revealed in Table 3 suggest that truncated anchors gave more complex patterns (higher *PC*) and more polymorphic bands able to distinguish between cultivars (higher *PP*) while the complete removal of the anchor from the primer sequence led to the disappearance of polymorphism from within the fingerprinting patterns.

We designed a group of primers harbouring anchors with low sequence complexity (3PCTanS, PCT1an3S, PCT2an3S, 3PCTanW, PCT1an3W, PCT2an3W, where S stands for G,C and W for A,T partial sequence degeneration of primer anchors) giving finally $PC_t = 32$ and $PP_t = 0$ with $PC_a = 5{\cdot}3$ (var. coeff. = 121·9 %). Primers with a S-degenerated anchor had substantially higher *PC* ($PC_{tS} = 27$) than a W-degenerated variant ($PC_{tW} = 6$). The results obtained are in accordance with the preliminary

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Table 3. *Effect of anchor length within a primer on quality of fingerprinting pattern*

Primer group	Total number of bands in patterns (PC_t)	Number of polymorphic bands in patterns (PP_t)
Without anchor (PCT1noan + PCT2noan)	12	0
3-bp anchor (3PCT1 + 3PCT2)	65	11
7-bp anchor (PCT1 + PCT2)	62	10

expectation, that S (GC degeneracy) anchors will fix primers more efficiently to templates than W (AT degeneracy) anchors thus yielding higher pattern complexity (higher *PC*). However, no new polymorphism could be generated experimentally.

We also studied the effect of 3′ anchor position on pattern complexity and polymorphism on primers with [GA] (PCT1) and [CT] (PCT2) core sequence (set of primers PCT1an3W, PCT1an3S, and PCT2an3W, PCT2an3S), where $PC_{t\ [GA]} = 17$ while $PC_{t\ [CT]} = 0$. We deduced, that core sequence type ([GA] versus [CT]) and not anchor sequences critically influenced final pattern complexity but no new polymorphism was generated.

Finally, we designed two primers with sequences reflecting the high theoretical frequencies of microsatellite loci within a plant genome (Lagercrantz *et al.* 1993; Wang *et al.* 1994; Hancock 2000). These primers had fully degenerated anchors (primers PCTAT and PCTAA). In this group $PC_t = 2$ and $PP_t = 0$. Primer PCTAT gave only two scorable bands, which were monomorphic throughout all eight cultivars. Primer PCTAA gave no scorable bands at all.

Table 4. *Potential of primers to generate highly informative fingerprinting patterns*

Primer	Number of different microsatellite patterns per primer	Polymorphic Information Content (*PIC*)
PCT1	6	0·929
PCT2	4	0·750
PCT3	1	0·000
PCT4	2	0·536
PCT5	2	0·429
PCT6	8	1·000
3PCT1	4	0·643
3PCT2	3	0·679
3PCT4L	4	0·492
3PCT5L	2	0·250
3PCTanS	2	0·250

The parameter of polymorphism information content *PIC* was calculated according to Botstein *et al.* (1980): $PIC_j = n(1-\Sigma P_{ij}^2)/(n-1)$ where n is the sample size, and P_{ij} the frequency of the i-th pattern revealed by the microsatellite primer j summed across all patterns revealed by the primer j.

In summary the complete set of 25 microsatellite primers applied to the flax genome gave a total number of bands within all patterns of $PC_t = 294$ and a total number of polymorphic bands per all patterns of $PP_t = 43$, which represents 14·6% polymorphic bands. We can compare these results with Godwin *et al.* (1997) who obtained 13·1% and 31·0% polymorphic bands for ISSR fingerprinting of banana and *Sorghum*, respectively. Such a comparison suggests that the percentage of polymorphic bands generated depends on the type of genome. The highest number of bands per pattern (sum of bands from all patterns generated under various annealing temperatures) was obtained by primer 3PCT1 having $PC = 36$. The highest number of polymorphic bands for all patterns generated was obtained by primer 3PCT2 ($PP = 7$) followed by primer 3PCT4L ($PP = 6$).

The most promising result with respect to cultivar recognition does not correspond to the highest *PP* values but is properly reflected by the *PIC* parameter (Table 4). The parameter *PIC* directly corresponds to the power of a microsatellite primer to discriminate between cultivars. While $PIC_{max} = 1$ corresponds to the patterns of all eight cultivars being different $PIC_{min} = 0$ represents a monomorphic pattern common to all eight cultivars. The highest discrimination was achieved by primer PCT6 having $PP = 5$ with $PIC = 1{\cdot}000$. This pattern is thus suitable for construction of a genomic library specifically enriched for [GATA] flax microsatellite sequences following a general strategy of rapid construction of MS-enriched genomic libraries (Brachet *et al.* 1999). The second best MS primer PCT1 ($PIC = 0{\cdot}929$) was able to distinguish six out of the eight cultivars.

A total of 38 various microsatellite patterns were generated with an average of 3·45 patterns per primer and average *PIC*: $PIC_a = 0{\cdot}542 \pm 0{\cdot}178$ ($P \leqslant 0{\cdot}05$). We can compare the value of *PIC* obtained with the results from 16 specific SSR loci of wild barley (Davila *et al.* 1999). From the barley data we can calculate average *PIC*: $PIC_a = 0{\cdot}855 \pm 0{\cdot}092$ ($P \leqslant 0{\cdot}05$); the higher value of *PIC* compared to our data corresponds to the application of SSR-flanking primer

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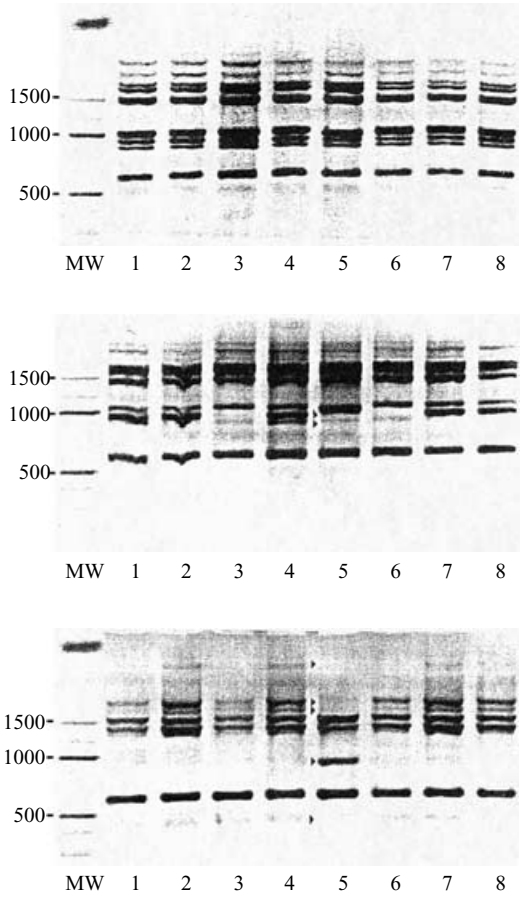

Fig. 3. The appearance of new polymorphic bands (arrows) at increasing annealing temperatures 55 °C, 60 °C and 65 °C, in *a*, *b*, *c*, respectively, for primer 3PCT2 (5′- VRV $[CT]_{15}$) leads to new polymorphism within flax microsatellite patterns.

pairs which are the product of a classical highly time-consuming fingerprinting strategy.

We also found that variation in PCR annealing temperature may be an important factor in generating new band polymorphisms. Increasing the annealing temperature within a temperature profile helped to generate new polymorphisms within the flax microsatellite patterns of primer 3PCT2. We succeeded in generating new polymorphic bands when increasing the annealing temperature from 55 °C ($PP = 0$, Fig. 3*a*) to 60 °C ($PP = 2$, Fig.3*b*), and to 65 °C ($PP = 5$, Fig. 3*c*). It is probable that cases of mispairing with the primer-template were reduced with an increase of annealing temperature. The recommended annealing temperatures for individual MS primers are given in Table 5.

No increase of pattern polymorphism was found using the strategy of pre- or post-PCR cutting by

Table 5. *Optimal annealing temperature found for each primer*

Primer	Annealing temperature (°C)
PCT1	65
PCT2	60
PCT3	60
PCT4	60
PCT5	55
PCT6	55 (with reamplification step)
3PCT1	65
3PCT2	65
3PCT4L	65
3PCT5L	60

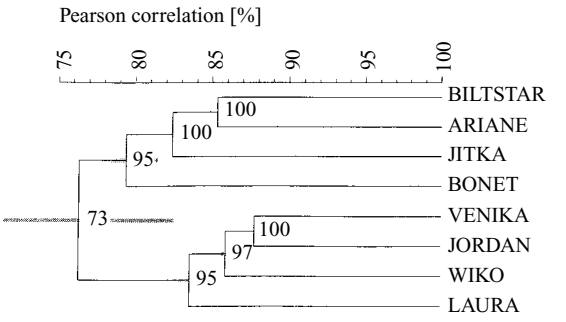

Fig. 4. Cluster analysis of flax cultivars based on microsatellite fingerprinting data was calculated by GelCompar II, from which the Pearson correlation coefficient and UPGMA clustering procedure were selected. Shadow bars correspond to error flags and digits on the cluster roots correspond to cophenetic correlation coefficient.

*Mse*I restriction endonuclease (New England BioLabs, USA) even though restriction digestion was reported to have a significant effect on MS pattern composition (Becker & Heun 1995).

Results from the cluster analysis based on microsatellite data are given in Fig. 4. The group of eight model cultivars was clearly clustered into two more homogeneous subclusters within which cultivars displayed only continuous differences. In order to evaluate clustering significance, error flags were calculated which are displayed as shadow bars (constructed using average similarity and related standard deviation) in Fig. 4. The smaller the error flag the more consistent a subcluster group is. Further, cophenetic correlation was calculated to express the consistency of a cluster (digits on the root of each cluster). Cophenetic correlation at the main root of the cluster CC_t corresponds to the value of the whole dendrogram: $CC_t = 76{\cdot}34\,\% \pm 4{\cdot}77\,\%$. It is in agreement with previous experiments (Siffelova *et al.* 1997, 1998) where cultivar clustering based on molecular data reliably split up analysed cultivars into several

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homogeneous subgroups within which only smooth transitions between cultivars existed without any clustering tendencies.

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REFERENCES

Aldrich, J. & Cullis, C. A. (1993). RAPD analysis in flax: Optimization of yield and reproducibility using KlenTaq1 DNA polymerase, Chelex 100, and gel purification of genomic DNA. *Plant Molecular Biology Reporter* **11**, 128–141.

Becker, J. & Heun, M. (1995). Mapping of digested and undigested random amplified microsatellite polymorphisms in barley. *Genome* **38**, 991–998.

Botstein, D., White, R. L., Scolnick, M. & Davis, R. W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphism. *American Journal of Human Genetics* **32**, 314–331.

Brachet, S., Jubier, M. F., Richard, M., Jung-Muller, B. & Frascaria-Lacoste, N. (1999). Rapid identification of microsatellite loci using 5′ anchored PCR in the common ash *Fraxinus excelsior*. *Molecular Ecology* **8**, 160–163.

Charters, Y. M., Robertson, A., Wilkinson, M. J. & Ramsay, G. (1996). PCR analysis of oilseed rape cultivars (*Brassica napus* L. ssp. *oleifera*) using 5′-anchored sequence repeat (SSR) primers. *Theoretical and Applied Genetics* **92**, 442–447.

Congiu, L., Chicca, M., Cella, R., Rossi, R. & Bernacchia, G. (2000). The use of random amplified polymorphic DNA (RAPD) markers to identify strawberry varietes: a forensic application. *Molecular Ecology* **9**, 229–232.

Cullings, K. W. (1992). Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. *Molecular Ecology* **1**, 233–240.

Cullis, C. A. & Govindaraju, D. (1992). Identification of RAPD polymorphisms among introgressed flax lines containing the M rust resistance genes, and the cloning of the PCR products. In *Proceedings of Plant Genome I Conference*. Town & Country Conference Center, San Diego, CA.

Cullis, C. A., Oh, T. J. & Gorman, M. B. (1995). Genetic mapping in flax (*Linum usitatissimum*). In *Proceedings of Breeding for Fiber and Oil Quality in Flax*, pp. 161–169. St. Valery en Caux, France: Centre Technique pour l'Etude et l'Amelioration du Lin.

Davila, J. A., Loarce, Y., Ramsay, L., Waugh, R. & Ferrer, E. (1999). Comparison of RAMP and SSR markers for the study of wild barley genetic diversity. *Hereditas* **131**, 5–13.

Fisher, P. J., Garnder, R. C. & Richardson, T. E. (1996). Single locus microsatellites isolated using 5′ anchored PCR. *Nucleic Acids Research* **24**, 4369–4371.

Godwin, I. D., Aitken, E. A. B. & Smith, L. W. (1997). Application of inter simple sequence repeat (ISSR) markers to plant genetics. *Electrophoresis* **18**, 1524–1528.

Gorman, M. & Parojcic, M. (1992). Genomic mapping in flax (*Linum usitatissimum*). In *Proceedings of Plant Genome I Conference*. Town & Country Conference Center, San Diego, CA.

Gorman, M. B., Cullis, C. A. & Alldridge, N. (1993). Genetic and linkage analysis of isozyme polymorphisms in flax. *Journal of Heredity* **84**, 73–78.

Gupta, M., Chyi, Y-S., Romero-Severson, J. & Owen, J. L. (1994). Amplification of DNA markers from evolutionary diverse genomes using single primers of simple-sequence repeats. *Theoretical and Applied Genetics* **89**, 998–1006.

Hancock, J. M. (2000). Microsatellites and other simple sequences: genomic context and mutational mechanisms. In *Microsatellites. Evolution and Applications* (Eds D. B. Goldstein & C. Schlotterer), pp. 1–9. Oxford: Oxford University Press.

Kantety, R. V., Zeng, X. P., Bennetzen, J. L. & Zehr, B. E. (1995). Assessment of genetic diversity in dent and popcorn (*Zea mays* L.) inbred lines using inter-simple sequence repeat (ISSR) amplification. *Molecular Breeding* **1**, 365–373.

Klocke, M., Frei, U. & Wenzel, G. (2000). Genome analysis of flax (*Linum usitatissimum* L.). *http://www.weihenstephan.de/pbpz/mm/mk1/mk1.htm*.

Lagercrantz, U., Ellegren, H. & Andersson, L. (1993). The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. *Nucleic Acids Research* **21**, 1111–1115.

Morgante, M. & Olivieri, A. M. (1993). PCR-amplified microsatellites as markers in plant genetics. *The Plant Journal* **3**, 175–182.

Siffelova, G., Pavelkova, M., Klabouchova, A., Wiesner, I. & Nasinec, V. (1997). Computer-aided RAPD fingerprinting of accessions from the ryegrass-fescue complex. *Journal of Agricultural Science, Cambridge* **129**, 257–265.

Siffelova, G., Pavelkova, M., Klabouchova, A., Wiesner, I., Nasinec, V. & Nasinec, I. (1998). RAPD fingerprinting of diploid *Lolium perenne* × hexaploid *Festuca arundinacea* hybrid genomes. *Biologia Plantarum* **40**, 183–192.

Smith, J. S. C., Chin, E. C. L., Shu, H., Smith, O. S., Wall, S. J., Senior, M. L., Mitchell, S. E., Kresowich, S. & Ziegle, J. (1997). An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): comparisons with data from RFLPs and pedigree. *Theoretical and Applied Genetics* **95**, 163–173.

Smith, S. (1998). Cultivar identification and varietal protection. In *DNA markers: Protocols, Applications and Overviews* (Eds G. Caetano-Annoles & P. M. Gresshoff), pp. 383–400. New York: VCH.

Spielmeyer, W., Lagudah, E. S., Singh, S. & Green, A. G. (1995). Identification and application of molecular markers in Linola breeding. In *Proceedings of the 3rd International Flax Breeding Research Group*, St Valery en Caux, France.

Spielmeyer, W., Green, A. G., Bittisnich, D., Mendham, N. & Lagudah, E. S. (1998). Identification of quantitative trait loci contributing to *Fusarium* wilt resistance on an AFLP linkage map of flax (*Linum usitatissimum*). *Theoretical and Applied Genetics* **97**, 633–641.

Thomas, M. R., Cain, P. & Scott, N. S. (1994). DNA typing of grapevines: A universal methodology and database for describing cultivars and evaluating genetic relatedness. *Plant Molecular Biology* **25**, 939–949.

Vogel, J. M. & Scolnik, P. A. (1998). Direct amplification

from microsatellites: detection of simple sequence repeat-based polymorphisms without cloning. In *DNA markers: Protocols, Applications and Overviews* (Eds Caetano-Annoles & P. M. Gresshoff), pp. 133–150. New York: VCH.

WANG, Z., WEBER, J. L., ZHONG, G. & TANKSLEY, S. D. (1994). Survey of plant tandem DNA repeats. *Theoretical and Applied Genetics* **88**, 1–6.

WEISING, K., ATKINSON, R. G. & GARDNER, C. (1995). Genomic fingerprinting by microsatellite-primed PCR: a critical evaluation. *PCR Methods and Applications* **4**, 249–255.

ZIETKIEWICZ, E., RAFALSKI, A. & LABUDA, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* **20**, 176–183.

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